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The DotL Protein, a Member of the TraG-Coupling Protein Family, Is Essential for Viability of *Legionella pneumophila* Strain Lp02

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Legionella pneumophila **is able to survive inside phagocytic cells by an internalization route that bypasses fusion of the nascent phagosome with the endocytic pathway to allow formation of a replicative phagosome. The *dot*/*icm* genes, a major virulence system of *L. pneumophila*, encode a type IVB secretion system that is required for intracellular growth. One Dot protein, DotL, has sequence similarity to type IV secretion system coupling proteins (T4CPs). In other systems, coupling proteins are not required for viability of the organism. Here we report the first example of a strain, *L. pneumophila* Lp02, in which a putative T4CP is essential for viability of the organism on bacteriological media. This result is particularly surprising since the majority of the *dot*/*icm* genes in Lp02 are dispensable for growth outside of a host cell, a condition that does not require a functional Dot/Icm secretion complex. We were able to isolate suppressors of the Δ*dotL* lethality and found that many contained mutations in other components of the Dot/Icm secretion system. A systematic analysis of *dot*/*icm* deletion mutants revealed that the majority of them (20 of 26) suppressed the lethality phenotype, indicating a partially assembled secretion system may be the source of Δ*dotL* toxicity in the wild-type strain. These results are consistent with a model in which the DotL protein plays a role in regulating the activity of the *L. pneumophila* type IV secretion apparatus.**

The gram-negative bacterium *Legionella pneumophila* is the causative agent of a potentially fatal form of pneumonia called Legionnaires' disease. *L. pneumophila* is found in freshwater environments, where it parasitizes many different species of protozoa (17). Humans become infected with *L. pneumophila* by inhaling aerosols generated from contaminated water sources. Upon entry into the human lung, *L. pneumophila* is internalized into bactericidal, alveolar macrophages. In contrast to phagosomes bearing most bacterial species, the compartment harboring *L. pneumophila* does not traffic into the lysosomal network and is not significantly acidified in the first few hours after uptake (26, 27). Instead, the phagosome interacts with early secretory vesicles at endoplasmic reticulum exit sites (29) and then undergoes a series of maturation events in which it sequentially associates with small vesicles, mitochondria, and eventually becomes surrounded by the rough endoplasmic reticulum (25, 60). Formation of this specialized compartment, called a "replicative phagosome," allows the microorganism to grow intracellularly (25, 28). Later in the infective cycle, a majority of the replicative phagosomes fuse with acidified compartments containing late endocytic markers, and this is believed to play an important role in the replicative cycle of this pathogen prior to exit from its host cell (59).

The key to *L. pneumophila*'s virulence is its ability to form a replicative phagosome, since mutants defective in this trait cannot replicate inside host cells and are thus unable to cause disease (24, 26). One large class of proteins that allow *L. pneumophila* to alter the endocytic pathway is encoded by the *dot*/*icm* genes (3, 5, 37). To date, over two dozen *dot*/*icm* genes have been identified and are clustered in two areas of the *L. pneumophila* chromosome (region I and region II) (63). Based on the similarity of the Dot/Icm proteins to proteins involved in conjugative DNA transfer, and the fact that the Dot/Icm system can transfer the mobilizeable plasmid RSF1010, it was proposed that the *dot*/*icm* genes of *L. pneumophila* encode a type IV secretion system (31, 50, 63).

Type IV secretion systems are able to export DNA and/or proteins out of the bacterial cell and include plasmid transfer systems (e.g., the *tra* and *trb* genes of the plasmid RP4), as well as systems involved in the delivery of virulence factors (10, 46, 66). The canonical type IV secretion system is encoded by the *virB* operon of the plant pathogen *Agrobacterium tumefaciens* (66). A number of other pathogens, including *Bartonella tribocorum*, *Bordetella pertussis*, *Brucella abortus*, *Helicobacter pylori*, and *Rickettsia prowazekii*, contain orthologues to the VirB proteins, and some of these systems have been shown to export proteins essential for virulence (10). In contrast to these type IV systems, the *L. pneumophila* Dot/Icm proteins have limited sequence similarity to the VirB proteins. Instead, the Dot/Icm proteins show high similarity to the transfer proteins from IncI plasmids (e.g., R64 and ColIb-P9) and compose a type IVB secretion system (31, 57).

As with most conjugative transfer systems, little is known about the specific function of many of the *L. pneumophila* Dot/Icm proteins. DotB was recently shown to possess ATPase activity and likely provides energy to the secretion apparatus

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(56). A second Dot protein, DotL, also contains a nucleotide binding motif and shows extensive sequence similarity to the conjugal transfer protein TrbC from IncI plasmids (19, 31). DotL also has detectable sequence similarity to a family of proposed ATPases known as TraG-like or type IV secretion system coupling proteins (T4CPs). The more notable members of the T4CP family include TraG (RP4 plasmid), TrwB (R388 plasmid), TraD (F plasmid), and the *A. tumefaciens* VirD4 protein (8, 18, 33).

The term "coupling protein" was proposed for this family because its members are believed to target, or couple, exported substrates to the secretion apparatus (8, 9, 15, 22, 23, 32, 61). This proposal was initially based on the phenotype of RP4 *traG* mutants, which were still able to process plasmid DNA into a secretion-competent intermediate and assemble a functional pilus but were unable to transfer the plasmid. This indicated that TraG plays a role in linking the two processes (9). Consistent with the idea of T4CPs linking substrates to the secretion apparatus, a number of T4CPs have been shown to interact with both exported substrates and with components of the secretion apparatus (2, 15, 20, 35, 61). Although T4CPs are absolutely required for export of substrates, their specific molecular function remains unknown (22).

We demonstrate here that a T4CP homologue, the DotL protein, is not only required for growth of *L. pneumophila* inside macrophages but is also essential for viability of certain strains on bacteriological media. The lethality caused by loss of *dotL* in those strains can be suppressed by mutations that inactivate the Dot/Icm complex, which is consistent with a DotL role in regulating the activity of this type IV secreton.

MATERIALS AND METHODS

Bacterial strains and media. All *L. pneumophila* strains used in the present study are derived from Lp02 (*hsdR rpsL thyA*) or JR32 (*hsdR rpsL*), two separate isolates of *L. pneumophila* Philadelphia-1 (3, 7, 38) (Table 1). *L. pneumophila* strains were cultured on *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES)-buffered charcoal yeast extract agar (CYET) or ACES-buffered yeast extract broth (AYET) supplemented with thymidine (100 μg/ml). Salt sensitivity was assayed on CYET plates containing 0.65% sodium chloride (11, 45, 64). Antibiotics (kanamycin, 20 μg/ml; chloramphenicol, 5 μg/ml; streptomycin, 50 μg/ml; gentamicin, 5 μg/ml) and sucrose (5%) were added as needed. *Escherichia coli* strains were cultured on Luria-Bertani medium, and antibiotics (kanamycin, 20 μg/ml; ampicillin, 100 μg/ml; chloramphenicol, 17 μg/ml) were added as needed. Replication-competent plasmids were propagated in the *E. coli* strain XL1-Blue. In order to propagate suicide plasmids containing the R6K origin of replication, a strain expressing the R6K π protein, *E. coli* strain DH5α(λ*pir*), was used (30, 67).

Plasmid construction. To make the Δ*dotL* suicide plasmid, pJB1001, two PCR-amplified fragments were cloned into the NotI/SalI sites of pSR47S (40). Fragment 1 was amplified by using the primers 5′-CCCAAACGGCCGCCAAA CGAGTATTTACCATGC (JVP201 with the EagI site underlined) and 5′-CCC AAAGGATCCCGCATCATGGCTCTAATTCC (JVP202 with the BamHI site underlined). Fragment 2 was amplified by using the primers 5′-CCCAAAGGA TCCGCTATTGGGCATGAAGAGAGC (JVP203 with the BamHI site underlined) and 5′-CCCAAAGTCGACCCTACTGATGCAACTTTAATCC (JVP204 with the SalI site underlined). Plasmid pJB1005 was constructed by inserting a gene encoding chloramphenicol acetyltransferase that was amplified from pKRP10 by using the primers 5′-CCCAAAGGATCCGAGGTTCCAACTTTCACC (JVP206 with the BamHI site underlined) and 5′-CCCAAAGGATCCCTGCCTTAAAAA AATTACGC (JVP207 with the BamHI site underlined) into the BamHI site of plasmid pJB1001.

To make the Δ*dotN* suicide plasmid, pJB3046, two PCR-amplified fragments were cloned into the NotI/SalI sites of pSR47S (40). Fragment 1 was amplified by using the primers 5′-CCCGCGGCCGCGGTGTATCGTTAGGTAAA ATGG (JVP289 with the NotI site underlined) and 5′-CCCGGATCCCGCCA TAGTTTGGTTCACATTCAGTC (JVP903 with the BamHI site underlined). Fragment 2 was amplified by using the primers 5′-CCCGGATCCGAGAAATG GGCTGCCAGTGC (JVP904 with the BamHI site underlined) and 5′-CCCGT CGACGCAGCTTTTAACTGATCGC (JVP286 with the SalI site underlined).

To make the Δ*dotM* suicide plasmid, pJB3050, two PCR-amplified fragments were cloned into the NotI/SalI sites of pSR47S (40). Fragment 1 was amplified by using the primers 5′-CCCGCGGCCGCGAAGCAATCTTCAGTCCTGG (JVP297 with the NotI site underlined) and 5′-CCCGGATCCCTGCTGTTGT TGTGCCATCTC (JVP901 with the BamHI site underlined). Fragment 2 was amplified by using the primers 5′-CCCGGATCCGATGAAGCGATTAGAGC TCTGG (JVP902 with the BamHI site underlined) and 5′-CCCGTCGACGCA TACAGAGAGTTATCTCC (JVP294 with the SalI site underlined).

pJB1010, the His-tagged version of DotL, was constructed by amplifying the *dotL* open reading frame (ORF) using plasmid pJB359 and the primers 5′-GA CATGCATGCGATGGGGTTGACTAATTAAGG (JVP217 with the SphI site underlined) and 5′-GACATGCATGCCCCGAAAGCAAAAGTTGCC (JVP218 with the SphI site underlined). The PCR product was digested with SphI and cloned into the SphI site of pQE-32 (Qiagen). The final construct can be used to express a fusion protein containing six histidines fused to amino acids 72 through 783 of DotL.

The *dotL* complementing clone, pJB1014, was constructed by first amplifying the *dotL* ORF from Lp02 chromosomal DNA by using the primers 5′-GGGGT ACCGGAATTAGAGCCATGATGCG (JVP227 with the KpnI site underlined) and 5′-GACATGCATGCGATGGGGTTGACTAATTAAGG (JVP217 with the SphI site underlined). The resulting product was digested with KpnI and SphI and ligated into KpnI/SphI-digested pJB908. pJB908, a derivative of the plasmid pKB5, has the following features: (i) an RSF1010 origin to permit replication in *L. pneumophila*, (ii) an Δ*oriT* mutation to prevent inhibition of growth in macrophages, and (iii) a *tac* promoter driving DotL expression (3). Constitutive expression from pJB1014 is able to rescue a *dotL* deletion strain for viability on plates and in macrophages and expresses similar levels of DotL compared to a wild-type strain.

pJB1242, the Δ*lvhB* suicide plasmid, was constructed by cloning two PCR-amplified fragments into the SalI and NotI sites of pSR47S. Fragment 1 was amplified by using the primers 5′-CCCGTCGACGTTTGGAGAAGTCAGTTT AAGG (JVP342 with the SalI site underlined) and 5′-CCCGGATCCTCATGG CGCCACCTTTTGC (JVP343 with the BamHI site underlined). Fragment 2 was amplified with the primers 5′-CCCGGATCCGAAGCACTCGAACTATA AACC (JVP344 with the BamHI site underlined) and 5′-CCCGCGGCCGCG TTTCGCCATTGTATCCC (JVP345 with the NotI site underlined).

pJB1304, containing the *lvhB* operon, was constructed by first amplifying the *lvhB* operon from JR32 chromosomal DNA by using the primers 5′-CCCGTC GACGTTTGGAGAAGTCAGTTTAAGG (JVP342 with the SalI site underlined) and 5′-CCCGCGGCCGCGTTTCGCCATTGTATCCC (JVP345 with the NotI site underlined). The resulting product was digested with SalI and NotI and ligated into SalI/NotI-digested pJB1300. pJB1300, a derivative of the plasmid pKB5 (3), has the HindIII site in the polylinker replaced with a unique NotI site.

Antibody production. pJB1010, a polyhistidine-tagged version of DotL in which the amino-terminal signal sequence of DotL was replaced with six histidines, was purified by using Ni-nitrilotriacetic acid chromatography (Qiagen). The purified His_6-DotL fusion protein was injected into rabbits to raise polyclonal antibodies against DotL (Cocalico). The serum recognized a single protein from wild-type *L. pneumophila* extracts that was absent in extracts from an *E. coli* strain and a *L. pneumophila* strain lacking the *dotL* gene.

Fractionation and Western analysis. *L. pneumophila* was fractionated as previously described (55). Briefly, a culture of Lp02 was grown to mid-exponential phase, and the cells were pelleted and resuspended in 50 mM Tris-HCl (pH 8.0), 0.5 M sucrose, 5 mM EDTA, and 0.1 mg of lysozyme/ml. The cell suspension was incubated on ice for 1 h, $MgSO_4$ was added to a final concentration of 20 mM, and spheroplasts were collected by centrifugation at 5,000 × *g*. The pellet was resuspended in 50 mM Tris-HCl (pH 8.0), sonicated, and then centrifuged at 5,000 × *g* to collect any unlysed cells. The supernatant was then centrifuged at 100,000 × *g* for 1 h at 4°C to obtain a total membrane fraction. The supernatant was removed, centrifuged at 100,000 × *g*, and saved as the cytoplasmic sample. The pellet was washed and resuspended in 50 mM Tris-HCl (pH 8.0). The inner membranes were solubilized by the addition of Triton X-100, and the outer membranes were collected by centrifugation at 100,000 × *g*. Fractions were resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and either Coomassie blue stained for total protein or transferred to a membrane and probed with the anti-DotL serum (1:5,000).

Cell culture. The histiocytic cell line U937 (American Type Culture Collection) was cultured in RPMI 1640 media (BioWhittaker) containing 10% fetal

TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant genotype[a]	Source or reference
Strains		
E. coli strains		
DH5α	*endA1 hsdR17* (r_K^- m_K^+) *glnV44 thi-1 recA1 gyrA* (Nal^r) *relA1* (Δ*lacIZYA-argF*)*U169 deoR* (ϕ80d*lac* Δ*lacZ*) M15	67
DH5α(λpir)	DH5α(λpir) *tet*::*Mu*	30
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F′ *proAB lacI*^{q} *Z*ΔM15 Tn*10* (*Tet*^{r})]	Stratagene
L. pneumophila strains		
Philadelphia-1	Wild-type strain	3, 38
Lp01	Philadelphia-1 *rpsL hsdR*	3
Lp02	Philadelphia-1 *rpsL hsdR thyA* mutant	3
Lp03	Lp02 *dotA* mutant	3
JR32	Philadelphia-1 *rpsL hsdR*	38
JV1001	Lp02 *dotL*$^+$/Δ*dotL*	This study
JV1003	Lp02 *dotL*$^+$/Δ*dotL*::Cm^r	This study
JV1067	JV1003 + pJB1014	This study
JV1630	JR32 Δ*lvhB*	This study
JV1631	JR32 Δ*lvhB*	This study
JV2114	JR32 Δ*dotL*	This study
JV2348	JR32 Δ*dotL* + pJB1079	This study
JV2349	JR32 Δ*dotL* + pJB1081	This study
JV2097	Lp02 *dotM*$^+$/Δ*dotM*::Cm^r	This study
JV2100	Lp02 *dotN*$^+$/Δ*dotN*::Cm^r	This study
JV2114	JR32 Δ*lvhB dotL*$^+$/Δ*dotL*::Cm^r	This study
JV2116	JR32 Δ*lvhB dotL*$^+$/Δ*dotL*::Cm^r	This study
JV2118	JR32 Δ*lvhB dotL*$^+$/Δ*dotL*::Cm^r	This study
JV2153	JR32 Δ*dotB*	This study
JV2238	Lp02 *dotM*$^+$/Δ*dotM*::Cm^r Δ*dotA*	This study
JV2240	Lp02 *dotN*$^+$/Δ*dotN*::Cm^r Δ*dotA*	This study
JV2256	Lp02 Δ*dotV dotL*$^+$/Δ*dotL*::Cm^r	This study
JV2258	Lp02 Δ*dotO dotL*$^+$/Δ*dotL*::Cm^r	This study
JV2260	Lp02 Δ*dotP dotL*$^+$/Δ*dotL*::Cm^r	This study
JV2262	Lp02 Δ*dotE dotL*$^+$/Δ*dotL*::Cm^r	This study
JV2264	Lp02 Δ*icmQ dotL*$^+$/Δ*dotL*::Cm^r	This study
JV2274	Lp02 Δ*icmX dotL*$^+$/Δ*dotL*::Cm^r	This study
JV2276	Lp02 Δ*dotA dotL*$^+$/Δ*dotL*::Cm^r	This study
JV2282	Lp02 Δ*icmS dotL*$^+$/Δ*dotL*::Cm^r	This study
JV2284	Lp02 Δ*icmR dotL*$^+$/Δ*dotL*::Cm^r	This study
JV2286	Lp02 Δ*dotB dotL*$^+$/Δ*dotL*::Cm^r	This study
JV2290	Lp02 Δ*dotU dotL*$^+$/Δ*dotL*::Cm^r	This study
JV2292	Lp02 Δ*icmF dotL*$^+$/Δ*dotL*::Cm^r	This study
JV2294	Lp02 Δ*citA dotL*$^+$/Δ*dotL*::Cm^r	This study
JV2465	Lp02 *dotM*$^+$/Δ*dotM*::Cm^r + *pdotL*$^+$	This study
JV3748	Lp02 Δ*dotH dotL*$^+$/Δ*dotL*::Cm^r	This study
JV3750	Lp02 Δ*icmT dotL*$^+$/Δ*dotL*::Cm^r	This study
JV3752	Lp02 Δ*icmW dotL*$^+$/Δ*dotL*::Cm^r	This study
JV3754	Lp02 Δ*dotI dotL*$^+$/Δ*dotL*::Cm^r	This study
JV3756	Lp02 Δ*dotJ dotL*$^+$/Δ*dotL*::Cm^r	This study
JV3758	Lp02 Δ*dotK dotL*$^+$/Δ*dotL*::Cm^r	This study
JV3760	Lp02 Δ*dotD dotL*$^+$/Δ*dotL*::Cm^r	This study
JV3762	Lp02 Δ*dotC dotL*$^+$/Δ*dotL*::Cm^r	This study
JV3765	Lp02 Δ*icmV dotl*$^+$/Δ*dotL*::Cm^r	This study
JV3767	Lp02 Δ*dotG dotL*$^+$/Δ*dotL*::Cm^r	This study
JV3769	Lp02 Δ*dotF dotL*$^+$/Δ*dotL*::Cm^r	This study
Plasmids		
pJB908	pKB5 Δ*oriT*	56
pJB921	Δ*dotB* in pSR47S	55
pJB1001	Δ*dotL* in pSR47S	This study
pJB1005	Δ*dotL*::Cm^r in pSR47S	This study
pJB1010	His-tagged DotL in pQE-32	This study
pJB1014	pJB908 + *dotL*$^+$	This study
pJB1079	pJB1014 + Gen^r cassette	This study
pJB1081	pJB908 + Gen^r cassette	This study
pJB1242	Δ*lvhB* in pSR47S	This study
pJB1300	pKB5 with *NotI* site	This study

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Relevant genotype[a]	Source or reference
pJB1304	*lvhB* operon in pJB1300	This study
pJB3046	Δ*dotN* in pSR47S	This study
pJB3050	Δ*dotM* in pSR47S	This study
pKB5	RSF1010 cloning vector	3
pSR47S	*oriR6K oriTRP4 kan sacB*	40

[a] Nal^r, nalidixic acid resistant; Tet^r, tetracycline resistant; Gen^r, gentamicin resistant.

bovine serum (BioWhittaker). Cells were differentiated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma) as described previously (3). Differentiated U937 cells were plated as a confluent monolayer in 24-well plates, with each well containing ca. 2×10^6 cells per well.

Southern blot analysis. *L. pneumophila* chromosomal DNA was isolated by a combination of a high-salt precipitation to eliminate contaminating proteins, followed by isopropanol precipitation of the DNA. Chromosomal DNA was digested with 10 U of HaeII restriction enzyme overnight at 37°C. Southern blots were performed according to the ECL Southern hybridization kit (Amersham), with probes specific to regions flanking *dotL* (from pJB1001) or *dotB* (pJB921).

Transposon mutagenesis of *L. pneumophila*. *L. pneumophila* was mutagenized by using the transposon delivery system encoded on pJK211-2 (13). pJK211-2 contains a temperature-sensitive origin that is not permissive for replication at 37°C, an altered sites transposase that increases the randomness of insertion, and a mini-Tn*10* transposon containing a kanamycin cassette (KanR) and a conditional origin from plasmid R6K later used to recover the transposon insertions in *E. coli* strain DH5α(λpir). A pool of insertions was placed on sucrose chloramphenicol plates to select for recombinants (sucrose to select for recombinants and chloramphenicol to select for the Δ*dotL*::Cm^r). Chloramphenicol-resistant (Cm^r), kanamycin-sensitive, sucrose-resistant (Suc^r) colonies were colony purified and scored for loss of the plasmid-encoded resistance cassette to ensure they had resolved the integrated plasmid. Insertions were recovered as previously described (13). The site of insertion was identified by sequencing by using the primers JVP348 (GGATCTGGTACCGGATCC) or JVP349 (TCAACAGGTT GAACTGCGGATC).

Screen for suppression of the Δ*dotL* lethality. Plasmids pJB1001 and pJB1005 were transferred into *L. pneumophila* strains by using an RP4 conjugation system encoded on pRK600 (14). *L. pneumophila* strains containing the integrated plasmid were selected by plating on CYET containing kanamycin and streptomycin. Resulting merodiploid strains that had a second crossover event were selected by plating on CYET plates containing 5% sucrose. Resolution of the integrated plasmid was confirmed by loss of kanamycin resistance. In the case of strains containing the Δ*dotL*::Cm^r cassette, sucrose-resistant colonies were streaked onto chloramphenicol to screen for the wild-type or mutant *dotL* alleles.

Replication of *L. pneumophila* strains in U937 cells. *L. pneumophila* strains were resuspended in phosphate-buffered saline to an optical density at 600 nm of 1. The bacterial suspensions were then diluted 1:1,000 in RPMI 1640 containing 10% fetal bovine serum, and 2 mM glutamine. A monolayer of TPA-treated U937 cells were infected with various *L. pneumophila* strains at a multiplicity of infection of one for 1 h. The monolayers were washed with fresh RPMI and then incubated in RPMI 1640 containing 10% fetal bovine serum and 2 mM glutamine at 37°C and 5% CO_2. Thymidine was added when appropriate. At 1, 24, 48, and 72 h postinfection, cells were lysed in sterile ddH_2O and dilutions were plated on CYET. Plates were incubated for 4 days at 37°C, and viable counts were determined.

Accession numbers. GenBank accession numbers for submitted sequences are as follows: DotU is AF533658 and DotV is AF533657.

RESULTS

DotL is an inner membrane protein with homology to T4CPs. The *L. pneumophila* DotL protein has extensive similarity to several proteins found in GenBank (Fig. 1A), with the highest degree of similarity (56% identity) to an uncharacterized ORF found in *Coxiella burnetii* that has been proposed to be part of a type IV secretion system (57). DotL also has similarity to TrbC (27% identity), a protein required for the transfer of the IncI plasmids R64 and ColIb-P9 (Fig. 1, top) (19, 31), and to an ORF on a plasmid found in *Pseudomonas syringae* strains that may be part of the conjugative transfer apparatus for this plasmid (57). DotL also has sequence similarity, extending primarily over the Walker A box, to members of the type IV coupling protein family, most notably TraD, TraG, TrwB, and VirD4 (Fig. 1A). In addition, DotL shares a number of characteristics with members of the T4CP family. These include a predicted size of 86 kDa, the presence of a potential nucleotide binding motif (a Walker A box), and an amino-terminal hydrophobic sequence that would likely target the protein to the bacterial inner membrane (52, 65). These characteristics, combined with its homology, suggest DotL may be a T4CP.

To confirm the subcellular localization of DotL, *L. pneumophila* extracts were prepared, fractionated, and the protein was detected by Western analysis with a DotL specific antibody. The DotL protein was primarily localized to the membrane fraction (Fig. 1B, lane 3). Moreover, the majority of the protein was Triton X-100 soluble, indicating it was likely to be in the inner membrane of the bacterial cell (Fig. 1B, lane 4) (48). In addition, a smaller cross-reacting species of ca. 75 kDa could be detected that localized completely to the cytoplasmic fraction (Fig. 1B, lane 2) and is consistent with a DotL breakdown product lacking the hydrophobic amino-terminal transmembrane domains.

DotL is essential for viability on bacteriological media. To investigate the function of the DotL protein, we attempted to delete the *dotL* gene from the chromosome of Lp02, a strain of *L. pneumophila* with an intact *dot*/*icm* system (3, 63). Previous attempts to delete *dot*/*icm* genes have been uniformly successful, indicating that the Dot/Icm complex is not required for viability on bacteriological media (1, 3, 63). To construct an in-frame deletion of the *dotL* gene, ca. 500 bp of DNA upstream and downstream adjacent to the *dotL* gene was cloned into the suicide vector pSR47S, generating plasmid pJB1001 (Fig. 2). The *dotL* deletion plasmid was electroporated into strain Lp02 and introduced onto the chromosome by selecting for a single crossover event generating a *dotL*/Δ*dotL* merodiploid strain. Merodiploids that had resolved were selected by plating on sucrose, a toxic compound for gram-negative organisms containing the counterselectable marker *sacB* (4). Resolution of the merodiploid should result in an equal proportion of strains containing either the wild-type copy of *dotL* or Δ*dotL* on the chromosome (Fig. 2).

Examination of 14 independent sucrose resistant recombinants derived from the *dotL*/Δ*dotL* merodiploid strain revealed no strains that lacked the wild-type copy of *dotL* (Fig. 3, top panel). In contrast, sucrose resistant recombinants derived from a similarly constructed *dotB*/Δ*dotB* merodiploid (55) re-

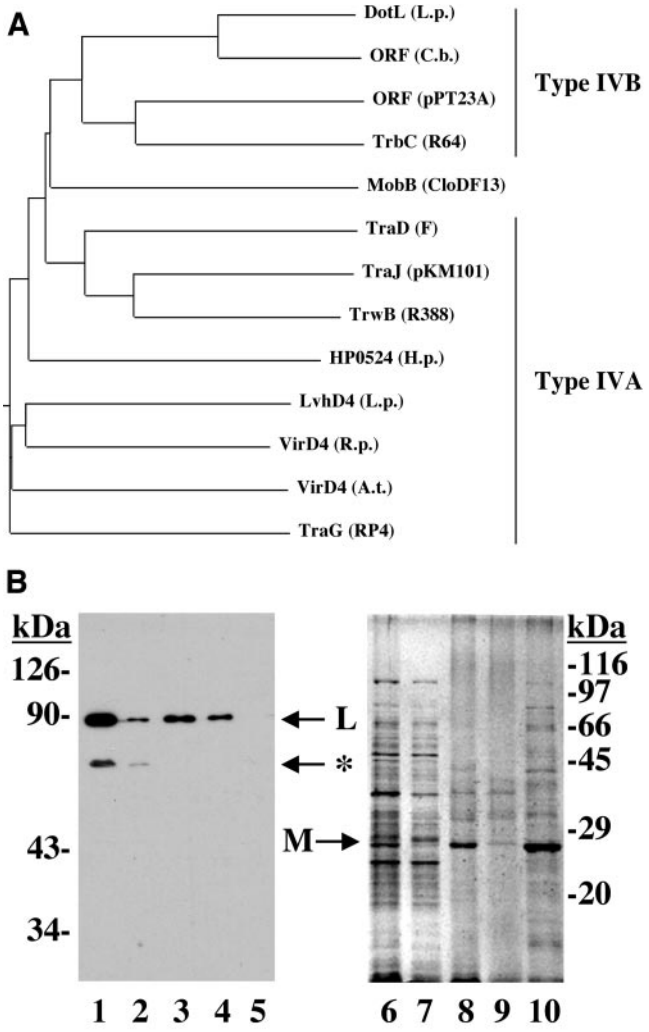

FIG. 1. (Top) DotL shows sequence similarity to members of the T4CP family. DotL has extensive similarity to a number of putative type IVB secretion system ATPases, including an uncharacterized ORF in *Coxiella burnetii* [ORF (C.b.)], the TrbC protein of the IncI plasmid R64 [TrbC (R64)], and a TrbC orthologue on the pPT23A plasmid of *Pseudomonas syringae* strains [ORF (pPT23A)]. DotL has similarity to plasmid T4CPs (MobB, TraD, TraJ, TrwB, and TraG from the plasmids CloDF13, F, pKM101, R388, and RP4, respectively) and to T4CPs from adapted conjugation systems found in pathogens (HP0524 from *Helicobacter pylori*, VirD4 from the Ti plasmid of *Agrobacterium tumefaciens*, and a VirD4 orthologue from *Rickettsia prowazekii*). Most strains of *L. pneumophila* contain at least one additional T4CP, LvhD4, which is part of a second type IV secretion system (51). The dendrogram was generated by using CLUSTAL W alignment. (Bottom) The DotL protein is localized to the inner membrane of *L. pneumophila*. Extracts of wild-type *L. pneumophila* were separated into cytoplasmic and membrane fractions by high-speed centrifugation. The membrane fractions were then further separated into inner membrane versus outer membrane fractions by extraction with the detergent Triton X-100. Duplicate samples were run on two 7.5% acrylamide gels; the first gel was transferred to a polyvinylidene difluoride membrane and probed with anti-DotL serum (lanes 1 to 5), whereas the second gel was stained with Coomassie blue for total protein (lanes 6 to 10). Lanes 1 and 6 are total cell lysates, lanes 2 and 7 are soluble cytoplasmic fractions, lanes 3 and 8 are total membrane, lanes 4 and 9 are Triton X-100 soluble (inner membrane), and lanes 5 and 10 are Triton X-100 insoluble (outer membrane). All samples were loaded proportionally except for lanes 8, 9, and 10, which were overloaded in order to detect the protein profile (lane 8 is 3-fold, lane 9 is 2-fold, and lane 10 is 25-fold overloaded relative to lanes 1 to 7). The quality of the fractionation procedure can be determined by monitoring the localization of the major outer membrane protein, MOMP, on the Coomassie blue-stained gel (lane 10) (44). A DotL breakdown product detected by Western analysis is indicated with an asterisk (lane 2).

sulted in ten strains containing wild-type *dotB* and four strains containing Δ*dotB* (Fig. 3, bottom panel). To ensure that the recombination event in the *dotL*/Δ*dotL* merodiploid strain was not theoretically impossible, recombinants were selected in a merodiploid containing the *dotL*$^+$ plasmid pJB1014. In this situation, chromosomal *dotL* deletions were recovered, indicating that a Δ*dotL* could be obtained in a strain exogenously expressing DotL (data shown below). Finally, the Δ*dotL*::Cm^r reporter plasmid pJB1005 could not be introduced directly onto the chromosome of the *L. pneumophila* strain Lp02 by using natural transformation (56), confirming the difficulty of constructing a *dotL* deletion. These results indicated a strong bias against deleting the wild-type version of *dotL* and suggested that loss of *dotL* may result in lethality of *L. pneumophila* on bacteriological media.

Although it appeared not to be feasible to isolate a strain lacking *dotL*, it was possible that an insufficient number of events were examined in order to identify such a strain. To screen a larger number of recombination events, the deletion strategy was repeated with a chloramphenicol-marked version of the *dotL* deletion. A *dotL*/Δ*dotL*::Cm^r merodiploid was subjected to selection on sucrose, in the absence of chloramphenicol, and the presence of the Δ*dotL*::Cm^r cassette was subsequently screened by plating sucrose resistant recombinants on medium containing chloramphenicol. Examination of a larger number of sucrose-resistant strains still failed to detect a recombinant that contained just the Δ*dotL*::Cm^r allele (0 of 753 events scored). Based on these results, we conclude that *dotL* is required for the viability of the *L. pneumophila* strain Lp02 on bacteriological media.

Isolation of suppressors of Δ*dotL*. In order to determine whether it was possible to suppress the lethality caused by loss of *dotL*, we plated an even greater number of the *dotL*/Δ*dotL*::Cm^r merodiploid on plates containing sucrose and chloramphenicol, thereby directly selecting for loss of *dotL*. Rare sucrose-resistant, chloramphenicol-resistant recombinants were isolated at a rate of $\sim 10^{-6}$. This was consistent with *dotL* being an essential gene, with the chloramphenicol-resistant colonies that arose being pseudorevertants due to spontaneous mutations in other genes. To identify the nature of the pseudorevertants, random transposon insertions were generated in the *dotL*/Δ*dotL*::Cm^r merodiploid strain background by using a mini-Tn*10* transposon, and the insertion pool was plated on sucrose and chloramphenicol to select for strains that could tolerate loss of *dotL*. Thirty-three such insertions were isolated from independent pools. These strains were first analyzed by Southern blot to ensure that they had only one insertion. To confirm that the phenotype was linked to the transposon insertion, the strains were recreated by transforming the transposon and flanking chromosomal DNA into the original, unmutagenized merodiploid strain by using natural transformation (56). Examination of the 33 strains by this

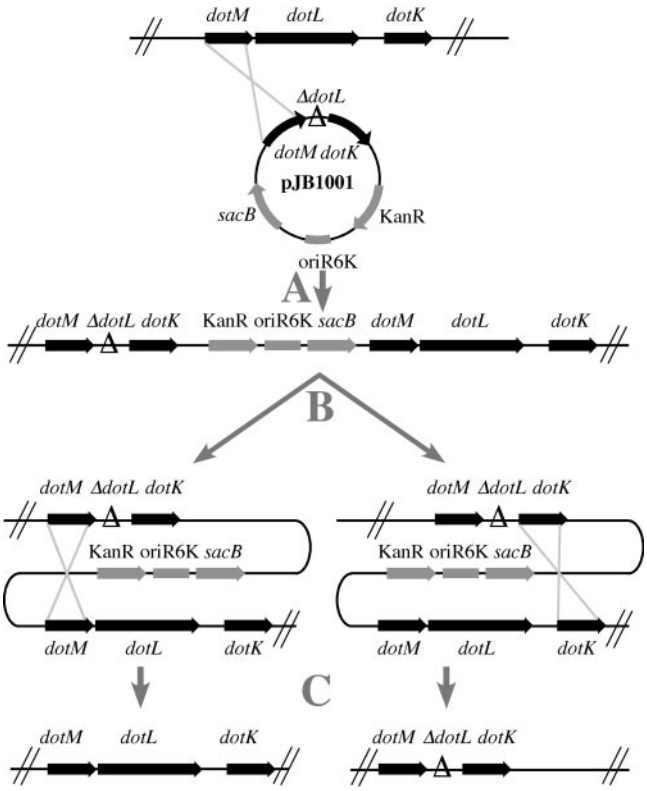

FIG. 2. Assay for ability of *L. pneumophila* to tolerate the Δ*dotL* mutation. A merodiploid consisting of a wild-type copy of *dotL* and a *dotL* deletion was constructed by integration of the suicide plasmid pJB1001. This plasmid contains an origin, from the R6K plasmid, that is unable to replicate in *L. pneumophila* strains lacking the replication protein π (30). pSR47S also contains the selectable marker, Kan^r, and a counterselectable marker, *sacB*, which confers sensitivity to sucrose. The kanamycin marker was used to select for a single crossover generating a merodiploid strain containing both *dotL*$^+$ and Δ*dotL* (step A). Recombination between duplicated sequences in the heterozygote was selected by growth on 5% sucrose (step B). If *dotL* is a nonessential gene, both *dotL*$^+$ and the Δ*dotL* will be obtained (step C). If *dotL* is an essential gene, then only wild-type *dotL* will be recovered.

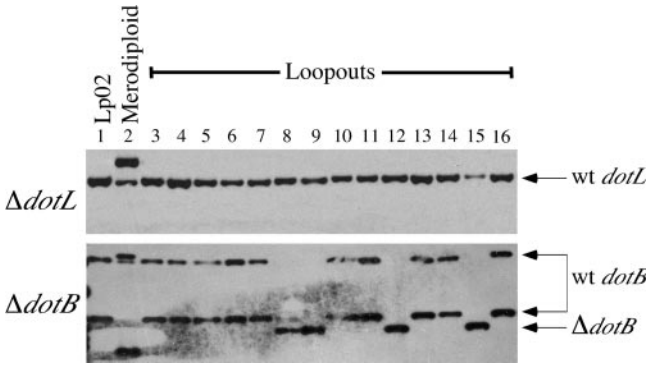

FIG. 3. *dotL* is an essential gene on bacteriological media. *dotL* and *dotB* merodiploids were constructed and recombinants selected as described in Fig. 2 were analyzed by Southern analysis (as described in Materials and Methods). The top panel shows a Southern blot of recombinants derived from a parental *dotL*/Δ*dotL* merodiploid strain probed with a 700-bp SalI fragment of DNA adjacent to the *dotL* gene. Lane 1 is the wild-type strain Lp02; lane 2 is JV1003, a Δ*dotL*/*dotL* merodiploid; and lanes 3 to 16 are JV1003 plated on CYET plus 5% sucrose. All 14 strains that were selected for sucrose resistance in this fashion retained the wild-type version of *dotL*. In contrast, the bottom panel is a similar experiment in which sucrose resistant recombinants derived from a *dotB*/Δ*dotB* merodiploid strain were probed with a *dotB*-region specific probe. Lane 1 is the wild-type strain Lp02; lane 2 is JV941, a *dotB*/Δ*dotB* merodiploid; and lanes 3 to 16 are JV941 selected on CYET plus 5% sucrose. In this case, two distinct types of recombinants are observed, a finding consistent with either *dotB* or Δ*dotB*, indicating that *dotB* is not an essential gene.

assay demonstrated that, in each case, the phenotype was linked to the transposon insertion. Finally, the transposons and flanking DNA were recovered on a plasmid, and the sites of the transposon insertions on the *L. pneumophila* chromosome were identified by sequencing off the end of each transposon.

Surprisingly, approximately one-half of the insertions (16 of 33) were in other *dot*/*icm* genes. This included four insertions in *dotA*, two in *dotG*, one in *dotI*, five in *dotO*, three in *icmF*, and one in *icmX* (Fig. 4). In most cases, the phenotype appeared to be due to inactivation of the gene the transposon was inserted in, because the insertions were in terminal genes of proposed operons (e.g., *dotA*, *dotO*, *icmF*, and *icmX*). Among the insertions that were not in known *dot*/*icm* genes, three mutants (JV1308, JV1343, and JV1499) were defective for intracellular growth of *L. pneumophila* when the insertions were separated from the Δ*dotL* (data not shown). The three mutants each contained an insertion in a different site of the same gene, which is located ca. 20 kb from region II (Fig. 4) (63). This gene codes for a small protein of 180 amino acids that has extensive homology to DotE (40% amino acid identity over 171 amino acids). We have designated this gene *dotV* because it is required for proper targeting of the *L. pneumophila* phagosome and for intracellular growth (unpublished results) (accession no. AF533657). Finally, the remaining insertions were not in known *dot*/*icm* genes or homologous genes and, when separated from the *dotL* deletion, caused the corresponding strains to exhibit various degrees of growth inhibition inside host cells (data not shown).

Other *dot*/*icm* mutations suppress loss of *dotL*. To confirm that loss of a specific *dot* gene could suppress a Δ*dotL*, we attempted to delete *dotL* in a strain containing an in-frame deletion of the *dotA* gene. In contrast to the previous attempt to delete *dotL* (Fig. 3), both *dotL* and Δ*dotL* loopouts were obtained from the Δ*dotA* *dotL*/Δ*dotL* merodiploid, demonstrating that loss of a single *dot* gene could allow the isolation of the Δ*dotL* mutation (Fig. 5). Because the Δ*dotL* suppressor hunt identified only a subset of *dot*/*icm* genes, we investigated whether they were the only *dot*/*icm* genes that, when inactivated, could suppress the Δ*dotL* lethality. In-frame deletions were constructed in 23 of the 26 *dot*/*icm* genes, and the Δ*dotL* suicide plasmid was integrated into each strain to assay for the ability to tolerate loss of *dotL*. Remarkably, *dotL* could be deleted in almost all of the strains containing different *dot*/*icm* mutations (Fig. 6). This suppression was specific in that *dotL* could not be deleted in a strain lacking a housekeeping gene found in region II, *citA*, which is not required for intracellular growth (Fig. 6) (42).

In contrast, inactivation of three *dot*/*icm* genes, *dotK*, *icmS*, and *icmW*, did not suppress loss of *dotL* (Fig. 6). *dotK* encodes an outer membrane protein with homology to OmpA and is only partially required for growth in amoebae (53). *icmS* and

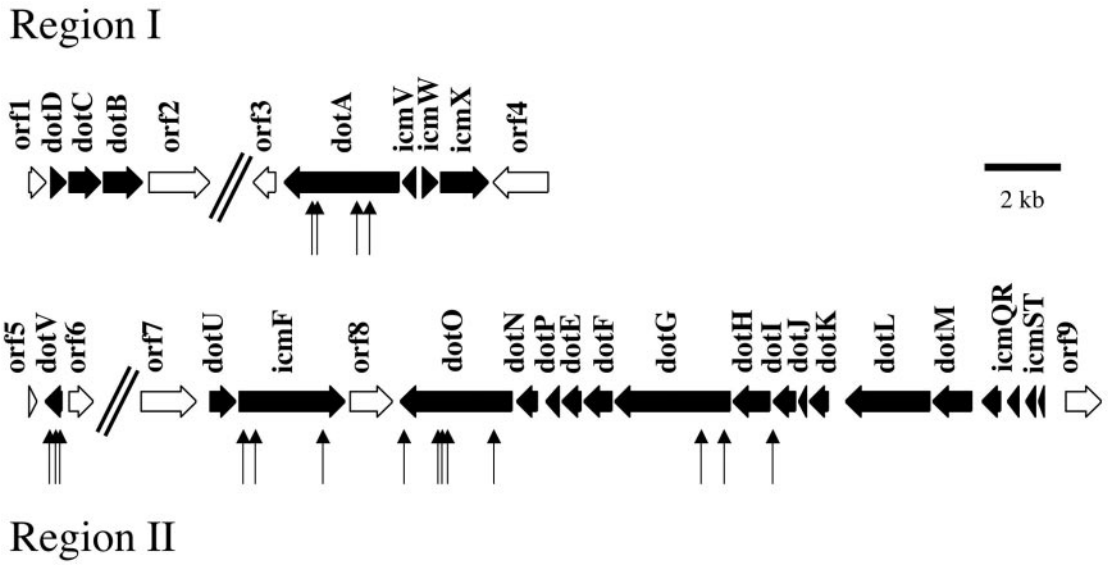

FIG. 4. Mini-Tn*10* insertions in multiple *dot*/*icm* genes suppress the lethality caused by loss of *dotL*. The *dotL*/Δ*dotL* merodiploid strain, JV1003, was mutagenized with mini-Tn*10*, and viable strains harboring Δ*dotL* were directly selected on sucrose-chloramphenicol-containing plates. Shown are the *L. pneumophila dot*/*icm* regions I and II (63). *dot*/*icm* genes are indicated with filled arrows, whereas flanking genes that are not required for intracellular growth are designated by open arrows (ORFs 1 to 9). Region I contains an 8-kb intervening region, which contains apparent housekeeping genes, separating the two *dot*/*icm* loci. *dotV* is separated from the rest of the *dot*/*icm* genes in region II by 20 kb. Mini-Tn*10* insertions that suppressed the Δ*dotL* lethality were found in *dotA*, *dotG*, *dotI*, *dotO*, *dotV*, and *icmF*, and the sites of insertions are indicated with vertical arrows.

icmW encode two small, acidic, cytoplasmic proteins that have been proposed to function as secretion chaperones (12). Similar to *dotK*, *icmS* and *icmW* are not absolutely required for the growth of *L. pneumophila* in permissive cell lines such as U937s and HL60s (12, 53). Therefore, it was possible that inactivation of these genes failed to suppress loss of *dotL* simply because they are not absolutely required for intracellular growth. However, inactivation of three other *dot*/*icm* genes—*icmF*, *dotU*, and *icmR*—did suppress loss of *dotL* (Fig. 6), even though loss of these genes caused only a partial inhibition of growth in permissive hosts (12, 53, 54, 62, 68). These results indicate that, although inactivation of the majority of the *dot*/*icm* genes can suppress the lethality caused by loss of *dotL*, there is specificity to the suppression.

***dotL* is not essential in all *L. pneumophila* strain backgrounds.** *dotL*, also known as *icmO*, is essential for viability in

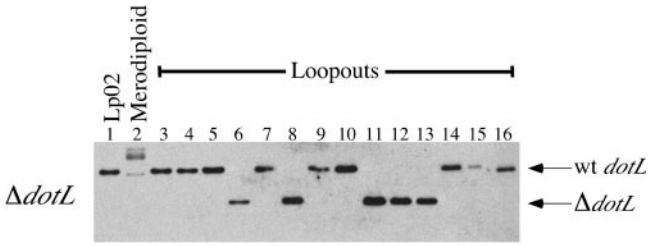

FIG. 5. The *dotL* gene can be deleted in a strain lacking *dotA*. Southern blot analysis of Δ*dotL* recombinants in a Δ*dotA* background. A 700-bp SalI fragment from pJB1001 encoding DNA flanking *dotL* on the chromosome was used as a probe to determine the status of *dotL* in these strains. Lane 1 is the wild-type strain Lp02; lane 2 is JV1005, a Δ*dotL*/*dotL* merodiploid in a *dotA* mutant background; and lanes 3 to 16 are JV1005 resolved on CYET plus 5% sucrose.

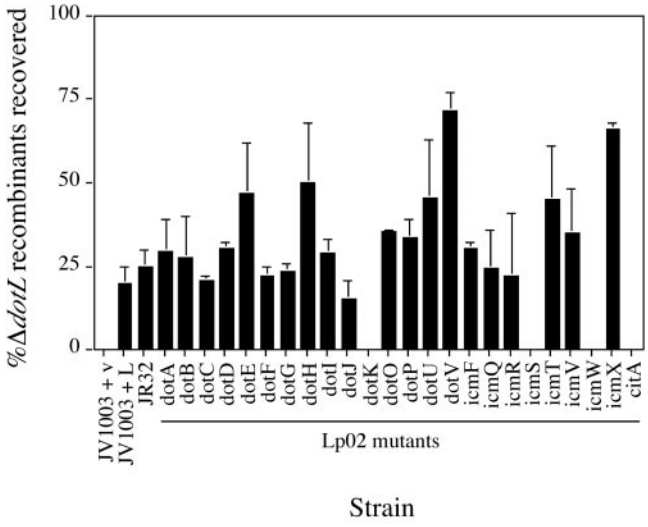

FIG. 6. Deletion of most *dot*/*icm* genes can suppress the lethality caused by deletion of *dotL*. A *dotL*/Δ*dotL*::CmR merodiploid was constructed in a variety of different *dot*/*icm* backgrounds. Sucrose-resistant recombinants were selected and then screened for the Δ*dotL* allele by resistance to chloramphenicol. Δ*dotL* recombinants could not be obtained from the *dotL*/Δ*dotL*::Cm^r merodiploid strain JV1003. The presence of a wild-type copy of *dotL* on a low-copy vector allowed the isolation of Δ*dotL* recombinants (JV1003 plus *dotL*). Inactivation of 20 of 23 *dot*/*icm* genes suppressed the loss of *dotL*. In addition, deletion of *citA*/*tphA*, a housekeeping gene found near the *dot*/*icm* genes, did not allow loss of *dotL* (42). In contrast, *dotL* is not essential in a related *L. pneumophila* strain, JR32. The data shown reflects the average number of Δ*dotL* recombinants recovered from scoring 50 events from four independent experiments.

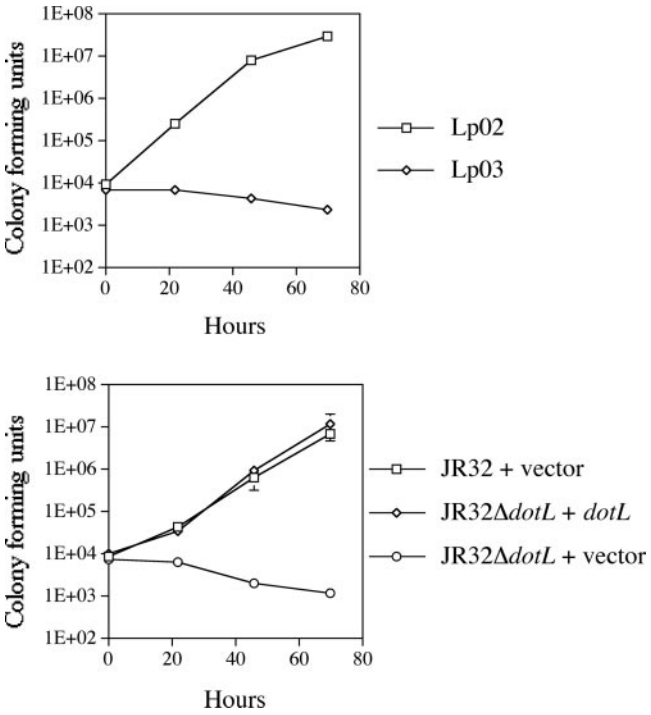

FIG. 7. *dotL* is required for growth of the *L. pneumophila* strain JR32 in U937 cells. A number of *L. pneumophila* strains were assayed for their ability to replicate inside U937 cells over 3 days. The top panel includes Lp02 (wild type) and Lp03 (a *dotA* mutant derivative of Lp02) as controls. The bottom panel includes JR32 containing the vector pJB908, a JR32Δ*dotL* strain containing the *dotL*$^+$ complementing clone pJB1014, and a JR32Δ*dotL* strain containing the vector pJB908. The data shown are the average of triplicate samples and are representative of two independent experiments.

the Lp02 background. However, it has been previously published that loss of *dotL* in another *L. pneumophila* strain, JR32, is not a lethal event (52). Lp02 and JR32 were independently derived from *L. pneumophila* Philadelphia-1, an organism isolated from the original Legionnaires' disease outbreak in 1976 (3, 38). Each strain was individually selected to be streptomycin resistant and to lack host restriction, and Lp02 was then also selected to be a thymidine auxotroph. Due to the relatedness of these two strains, it was surprising that *dotL*/*icmO* was essential for viability in Lp02 but was dispensable in JR32. To confirm that *dotL* was not an essential gene in JR32, a clean Δ*dotL* was constructed in the JR32 background and was indeed found to be viable on buffered CYE plates (data not shown). To examine intracellular growth, monolayers of U937 macrophages were challenged with wild-type *L. pneumophila* strains Lp02 and JR32. Both strains were able to multiply >1,000-fold in 3 days (Fig. 7). In contrast, an Lp02 strain lacking a functional *dotA* gene, Lp03, was unable to replicate inside macrophages. As previously shown, deletion of *dotL* in JR32 prevented the strain from replicating in U937 macrophages, and this defect could be complemented by the addition of a plasmid containing *dotL*$^+$ (Fig. 7) (52).

Although the JR32 Δ*dotL* strain was viable, closer examination revealed that it displayed a key difference from other *dot*/*icm* mutants (Table 2). Wild-type *L. pneumophila* stains, such as Lp02 and JR32, exhibit a significantly decreased plating

TABLE 2. Deletion of *dotL* in JR32 confers an enhanced salt sensitivity to the strain

Strain	Genotype	Plating efficiency[a] (%)	Phenotype
Lp02	Wild type	0.09	Salt sensitive
Lp03	Lp02 *dotA*	13	Salt resistant
JR32	Wild type	0.05	Salt sensitive
JV2153	JR32 Δ*dotB*	9.0	Salt resistant
JV2114	JR32 Δ*dotL*	0.002	Hyper-salt sensitive

[a] The plating efficiency was calculated as the number of colonies on a buffered CYE plate containing 0.65% sodium chloride divided by the number of colonies on a buffered CYE plate × 100.

efficiency on buffered CYE plates containing a low amount of sodium chloride (0.65%) compared to growth on plates lacking sodium chloride (11, 45, 64). Most *dot*/*icm* deletions (e.g., Δ*dotA*) exhibit an increased plating efficiency on plates supplemented with salt (Table 2). However, the JR32 Δ*dotL* strain was even more sensitive to sodium chloride than JR32 (Table 2), suggesting that the physiology of the JR32 Δ*dotL* is perturbed. These data suggest that loss of *dotL* in either the Lp02 strain or the JR32 strain is detrimental to the cell.

Mutation of *lvhD4* does not suppress the lethality caused by loss of *dotL*. Since the sequences of the *dot*/*icm* genes are identical between Lp02 and JR32, it is likely that there is an additional genetic difference between the two strains responsible for the more severe effect of deleting *dotL* in Lp02. For example, a gene may have been inactivated or lost during the derivation of JR32 that allows the JR32 Δ*dotL* strain to survive. Alternatively, a gene may be absent in Lp02 that is normally able to suppress the lethality caused by loss of *dotL*. In fact, a number of differences have been reported between various *L. pneumophila* serogroup I isolates including Lp01, the progenitor strain of Lp02, and JR32 (Table 1) (6, 36, 47). One potential candidate is *lvhD4*, which is present in JR32 but not in Lp01 (47). *lvhD4* is encoded in the *lvhB1-11*/*lvhD* operon and is a component of a second type IV secretion system found in *L. pneumophila* strains such as JR32 (51). *lvhD4* encodes a protein with similarity to T4CPs, most specifically to the *A. tumefaciens* VirD4 (51), and could in theory functionally substitute for DotL.

To confirm that the JR32 and Lp02 isolates we were working with contained and lacked *lvhD4*, respectively, we performed Southern analysis with a probe specific to *lvhD4* (Fig. 8, top). Consistent with previous reports, Lp01 and Lp02 lacked *lvhD4*, whereas JR32 and Philadelphia-1, the progenitor strain for both Lp02 and JR32, both contained it (Fig. 8A). Lp01 and Lp02 may have lost the *lvhB-lvhD* region during their derivation to become restriction minus, since a number of restriction or modification genes are located adjacent to the *lvhB*/*lvhD4* system (47). To determine whether there was a connection between the presence of *lvhD4* and Δ*dotL* lethality, we deleted *lvhD4* from JR32 or added it back to Lp02 and then assayed the consequence of deleting *dotL*. *dotL* could still be deleted in a JR32 strain lacking the *lvhB-lvhD4* region, indicating that *lvhD4* was not responsible for the viability of the JR32 Δ*dotL* strain (Fig. 8B). Likewise, the addition of the *lvhB –lvhD4* region from JR32 to the Lp02 strain did not suppress loss of *dotL*. Therefore, *lvhD4* does not appear to be responsible for

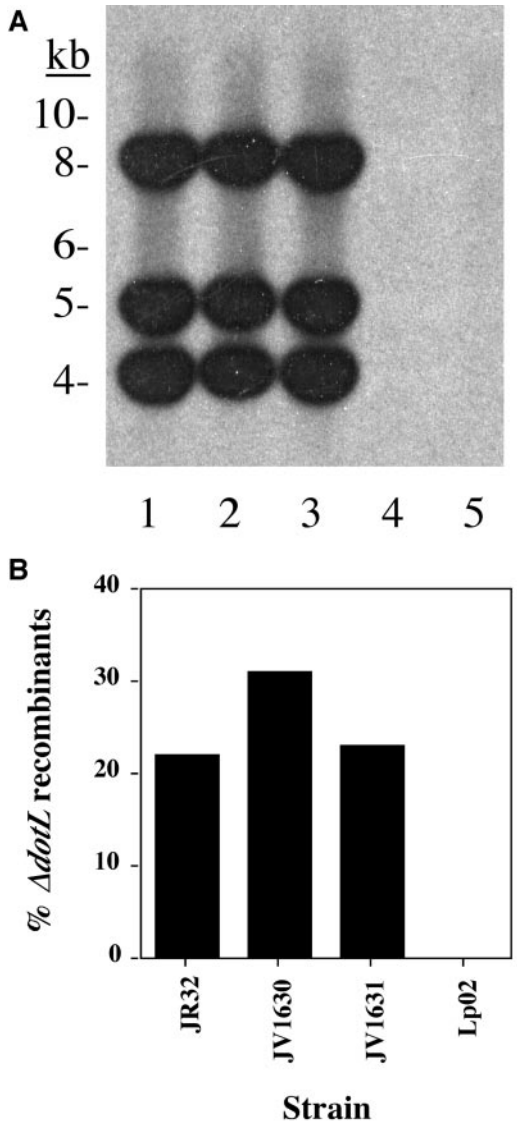

FIG. 8. The *lvhB* operon is not responsible for viability of the JR32 Δ*dotL* strain. (Top) The presence of the *lvhB*/*lvhD* operon in a variety of *L. pneumophila* strains was assayed by Southern analysis with a probe that contains the entire operon: lane 1 is the Philadelphia-1 progenitor of JR32, lane 2 is JR32, lane 3 is the Philadelphia-1 progenitor of Lp01 and Lp02, lane 4 is Lp01, and lane 5 is Lp02. (Bottom) A *dotL*/Δ*dotL* merodiploid of JR32 can be resolved to the Δ*dotL*, indicating it is not an essential gene. Two independently derived JR32 strains lacking the *lvhB* operon, JV1630 and JV1631, still allow deletion of *dotL*, whereas *dotL* is essential for viability in Lp02. The data shown reflect the average number of Δ*dotL* recombinants recovered from scoring 50 events from four independent experiments.

the altered requirements of *dotL* in these two *L. pneumophila* strains.

In addition to the *dot*/*icm* and the *lvhB* systems, certain *L. pneumophila* strains contain an additional type IV secretion system (6). This system is encoded in an ca. 65-kb locus, LpPI-1, that bears the hallmarks of a pathogenicity island. It contains homologues to a type IV secretion system that resembles the F plasmid, including a T4CP that resembles the F plasmid TraD protein, mobile genetic elements, and several

TABLE 3. *dotM* and *dotN* are required for the viability of Lp02

Strain	Genotype	No. of Δ*dotM* or Δ*doctN* recombinants/total[a]
JV2097	*dotM*$^+$/Δ*dotM*::Cm^r	0/600
JV2465	*dotM*$^+$/Δ*dotM*::Cm^r + *pdotL*$^+$	0/280
JV2238	*dotM*$^+$/Δ*dotM*::Cm^r Δ*dotA*	172/400
JV2100	*dotN*$^+$/Δ*dotN*::Cm^r	0/700
JV2240	*dotN*$^+$/Δ*dotN*::Cm^r Δ*dotA*	158/400

[a] This value was determined as the number of events in which the merodiploid resolved to either Δ*dotM*::Cm^r or to Δ*dotN*::Cm^r.

putative virulence factors (6). In contrast to *lvhD4*, LpPI-1 is present in Lp02 but is absent from JR32. However, deletion of the TraD-like protein from Lp02 did not suppress the lethality caused by loss of *dotL* (C. Vincent and J. P. Vogel, unpublished data). Thus, some additional, as-yet-uncharacterized, mutation must exist in one of these strains that is responsible for the differential requirement of *dotL* for viability.

***dotM* and *dotN* are also essential for viability in the Lp02 background.** While constructing a collection of *dot*/*icm* deletions, we were able to generate in-frame deletions in 23 of the 26 known *dot*/*icm* genes. However, similar to the *dotL* deletion, we could not construct a deletion in *dotM*, the gene upstream of *dotL* (Table 3). *dotM*, also known as *icmP*, codes for a predicted inner membrane protein with similarity to TrbA of the IncI plasmids R64 and ColIb-P9 (24% amino acid identity). Since *dotL* and *dotM* are likely to be cotranscribed in a two gene operon, *dotML*, it was possible that the lethality of the *dotM* deletion was due solely to polarity on the downstream *dotL* gene (Fig. 4). However, the Δ*dotM* mutation could not be obtained in the presence of a complementing clone containing a wild-type version of *dotL*, suggesting that the *dotM* lethality was not due to polarity but reflected the essentiality of *dotM* (Table 3). Moreover, insertions in *dotM* were obtained in a screen for genes that resemble *dotL*, i.e., genes that are essential in the presence of a functional Dot/Icm complex (13).

A third gene, *dotN*, also proved difficult to delete from the *L. pneumophila* chromosome. *dotN*, also known as *icmJ*, is located ca. 12 kb downstream of the proposed *dotML* operon and is the first gene of another predicted operon, *dotNO* (Fig. 4). *dotN* codes for a small protein of 208 amino acids that contains a high proportion of cysteines (4.3%). The lethality of the Δ*dotN* could not be due to simple polarity on the downstream gene *dotO* because deletions could easily be made in the *dotO* gene. Similar to *dotL*, both *dotM* and *dotN* could each be deleted in strains lacking a functional *dot* complex (Table 3). These results indicate that three *dot* genes, *dotL*, *dotM*, and *dotN* are each essential for viability on bacteriological media in the Lp02 background and in each case, the lethality can be suppressed by inactivation of the Dot/Icm complex.

DISCUSSION

The *dot*/*icm* genes are required for the intracellular replication of *L. pneumophila* and encode a type IVB secretion system that appears to have evolved from the conjugation apparatus of an IncI plasmid. We have demonstrated here that three *dot* genes, *dotL*, *dotM*, and *dotN*, are essential for growth of *L.*

pneumophila strain Lp02 on bacteriological media. This is in direct contrast to the established paradigm that the *dot*/*icm* genes are dispensable under the laboratory conditions of growth on plates (3, 37). In addition, we were able to isolate a large collection of suppressors of the Δ*dotL* lethality and have shown that the majority of these map to other *dot*/*icm* genes. However, inactivation of several *dot*/*icm* genes (*dotK*, *icmS*, and *icmW*) did not suppress loss of *dotL*, indicating specificity to the suppression.

DotL has limited homology to the T4CP family of proteins. T4CPs have been proposed to play a central role in type IV secretion systems (34). They have been shown to bind substrates synthesized in the cytoplasm and target them to the secretion apparatus in the inner membrane (2, 15, 61). T4CPs have also been shown to interact with other components of the secretion apparatus, namely, the VirB10-family of proteins (20, 35). Finally, T4CPs are absolutely required for export of substrates (22). Based on their homology to *Escherichia coli* FtsK and *Bacillus subtilis* SpoIIIE, and their ability to bind DNA, T4CPs have been proposed to function as molecular pumps, driving export of substrates via hydrolysis of ATP (22). In consideration of these traits, T4CPs would appear to be likely candidates to function as regulators of the type IV secretion complexes.

Based on the similarity of DotL to T4CPs, it is surprising that inactivation of the *dotL* gene in strain Lp02 is lethal. No other known T4CP is essential for viability. Moreover, the only proteins associated with conjugative transfer that that are required for bacterial viability are inhibitors of plasmid toxin segregation factors (43). DotL, however, shows no sequence similarity to such factors. In addition, if DotL functioned as an inhibitor of a plasmid segregation toxin, then the Δ*dotL* lethality suppressors would be predicted to map to the toxin. In contrast, many of the Δ*dotL* suppressors are components of the Dot/Icm machinery, and the non-*dot*/*icm* suppressors do not have homology to any known toxin inhibitors.

To explain these overall observations regarding toxicity induced by loss of *dotL*, we propose that loss of the DotL protein results in the accumulation of a toxic structure consisting of a portion of the Dot/Icm complex (Fig. 9). This partial Dot/Icm complex could be deleterious for a number of different reasons. First, a partial Dot/Icm complex could misassemble or misfold in the absence of DotL, disrupting the membrane in some fashion. Alternatively, loss of *dotL* could be toxic because the type IV secretion system forms an unregulated pore in the membrane in the absence of DotL (Fig. 9). In this model, DotL would play the role of a regulator of the complex, controlling the opening and closing of the pore.

We favor the unregulated pore model for the following reasons. First, if a misfolded subcomplex were the cause of the lethality one would not anticipate that inactivation of the majority of *dot*/*icm* genes (20 of 23) would suppress the loss of *dotL*. Second, the JR32 Δ*dotL* phenotype, increased sensitivity to sodium relative to a wild-type strain, is much more consistent with an unregulated pore. Although the sodium sensitivity of wild-type *L. pneumophila* strains is not well understood, it is believed to result from leakage of sodium ions through the Dot/Icm secretion apparatus (11, 64). This model is supported by the observation that strains resistant to sodium chloride often contain mutations in *dot*/*icm* genes (63). Taken in this context, loss of a regulator of the secretion pore is predicted to enhance the effect of exogenous sodium and is consistent with the hypersensitivity of the JR32 Δ*dotL*. Finally, there is precedence in the literature of an example in which loss of a protein resulted in an unregulated pore that can be lethal under certain circumstances. Inactivation of *Yersinia pestis lcrG* results in an unregulated type III secretion pore under certain conditions and has led to the model where LcrG forms a plug at the base of the apparatus (39, 58).

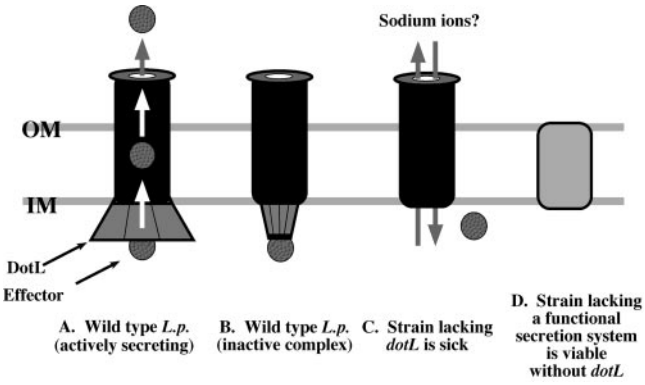

FIG. 9. Model for potential DotL regulation of the Dot/Icm translocator. (A) In wild-type *L. pneumophila* strains, the Dot/Icm proteins form a secretion apparatus in the membrane, which is used to export substrate(s). DotL is shown interacting with the complex as a hexameric gate based on homology to the hexameric T4CP, TrwB (21). Translocated substrates would be exported through the complex after interacting with DotL on the cytoplasmic face of the inner membrane. (B) During conditions in which *L. pneumophila* is not actively secreting substrates, the export apparatus would be closed via DotL and potentially substrates such as LidA (indicated as a ball) (13). (C) In the absence of DotL, the secretion pore might remain constitutively open and the cell would die, possibly due to cell lysis. (D) Inactivation of the Dot/Icm complex would suppress the Δ*dotL* lethality since an unregulated pore would no longer exist.

Based on the phenotype of a strain lacking *dotL*, mutations that cause lowered viability in the presence of an intact Dot/Icm apparatus were previously isolated (13). *lidA* was shown to encode a protein exported by the Dot/Icm system that may interact directly with DotL (13). Other *lid* genes may encode proteins necessary for proper assembly of the Dot/Icm complex, particularly a subcomplex consisting of DotL, DotM, and DotN. For example, three Lid proteins are involved in disulfide bond metabolism and, since the DotN protein is rich in cysteine residues, it may be that mutations affecting the formation of disulfide bonds could disrupt folding of DotN (13).

The Δ*dotL* lethality phenotype in Lp02 has proven to be useful for several additional reasons. First, it has provided a convenient plate selection for additional *dot*/*icm* mutants. This is noteworthy because many of the *dot*/*icm* genes were identified by labor-intensive screens that have never been performed to saturation (1, 3, 45). The only selection for *dot*/*icm* mutants previously available was based on the phenomenon that sodium-resistant *L. pneumophila* strains were often avirulent, although this phenomenon is poorly understood and may be mutagenic (11, 64). The benefit of our new selection is amply demonstrated since we have already identified an additional *dot*/*icm* gene, *dotV*, by this procedure.

The Δ*dotL* lethality phenotype also provides information

about existing Dot/Icm proteins. A number of Dot/Icm proteins that appear to be primarily cytoplasmic and not membrane associated were still able to suppress the loss of DotL when their genes were inactivated. For example, IcmQ and IcmR have been shown to be soluble proteins in the cytoplasm of *L. pneumophila* where IcmR appears to function as a chaperone for IcmQ (16). Although the specific function of IcmQ remains unknown, the fact that Δ*icmQ* and Δ*icmR* were able to suppress the lethality caused by the Δ*dotL* suggests that they are directly required for the assembly or activity of the Dot/Icm complex. Another example is the DotB ATPase (55). Although DotB does not appear to be an integral component of the Dot/Icm membrane complex, it is required for expression of the Δ*dotL* lethality trait, thus indicating that the protein plays a role in the assembly and/or function of the apparatus.

In contrast, inactivation of *icmS* or *icmW* did not suppress loss of *dotL*. Since *icmS* and *icmW* are predicted to encode cytoplasmic proteins and have been proposed to function as chaperones for secreted substrates (12), their failure to suppress is consistent with our model. Moreover, inactivation of a secreted substrate *ralF* (41) also failed to suppress loss of *dotL* (unpublished results). One additional Dot/Icm protein, the putative lipoprotein DotK, was also not required for Δ*dotL* lethality. Combined with the observation that a Δ*dotK* strain shows only mild defects for intracellular growth (53), this suggests that DotK is not essential for the formation of the Dot/Icm complex. Further examination of how various *dot*/*icm* mutants are able to suppress loss of *dotL* may reveal information on which components are key to formation of the secretion pore.

A third interesting observation that resulted from our analysis of the Δ*dotL* lethality involved *dotM* and *dotN*. Similar to *dotL*, we discovered that *dotM* and *dotN* are also essential for viability in the Lp02 background and are not essential for the viability of JR32 on bacteriological media but are required for growth of JR32 inside macrophages (50). Since all three proteins appear to code for inner membrane components of the secretion apparatus, it is possible that DotM and DotN interact with DotL and regulate its activity, perhaps by modulating its proposed nucleotide hydrolysis capability. In fact, we have recently shown that DotM can be coimmunoprecipitated by using DotL specific antibodies (Vincent and Vogel, unpublished).

It is interesting that deleting *dotL* in two very closely related strains results in very different phenotypes: death versus life. This is likely to be due to a genetic difference between the two strains acquired during their derivation. The JR32 strain may have acquired a suppressor mutation or Lp02 may have lost a gene that prevents Δ*dotL* lethality. One difference between these strains is that Lp02 lacks the second type IV secretion system encoded by the *lvhB* operon (47). However, deletion of the *lvhB* operon in JR32 did not cause the *dotL* deletion to be lethal, and therefore the identity of the suppressor(s) remains to be discovered. Nevertheless, the difference between these two strains may not be as profound as it initially appeared, since the JR32 Δ*dotL* strain is less fit than a wild-type strain, as demonstrated by its hyper-NaCl sensitivity. It is possible that the difference in phenotypes between the two strains is more a matter of degrees of sensitivity to loss of *dotL* rather than JR32 being impervious to its loss.

The Δ*dotL* phenotype described here is consistent with the proposal that T4CPs function as inner membrane gates for exported substrates (49). Further characterization of this interesting phenomenon should shed light not only on the function of DotL and other T4CPs but also on the *L. pneumophila* Dot/Icm complex and other type IV secretion systems.

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